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Model-based rationalization of mixture toxicity and accumulation in *Triticum aestivum* upon concurrent exposure to yttrium, lanthanum, and cerium

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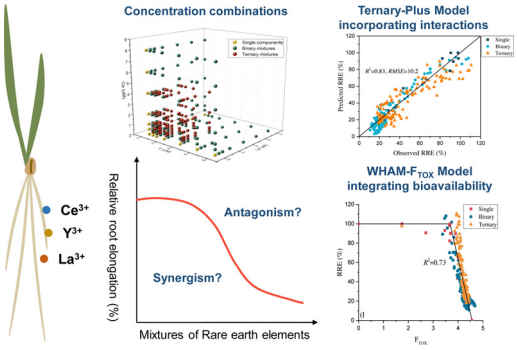
GRAPHICAL ABSTRACT



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ABSTRACT

Rare earth elements (REEs) often co-exist in the environment, but predicting their 'cocktail effects' is still challenging, especially for high-order mixtures with more than two components. Here, we systematically investigated the toxicity and accumulation of yttrium, lanthanum, and cerium mixtures in *Triticum aestivum* following a standardized bioassay. Toxic effects of mixtures were predicted using the reference model of Concentration Addition (CA), Ternary model, and Ternary-Plus model. Interactions between the REEs in binary and ternary mixtures were determined based on external and internal concentrations, and their magnitude estimated from the parameters deviated from CA. Strong antagonistic interactions were found in the ternary mixtures even though there were no significant interactions in the binary mixtures. Predictive ability increased when using the CA model, Ternary model, and Ternary-Plus model, with R^2 = 0.78, 0.80, and 0.87 based on external exposure concentrations, and R^2 = 0.72, 0.73, and 0.79, respectively based on internal concentrations. The bioavailability-based model WHAM-F_{TOX} explained more than 88 % and 85 % of the toxicity of binary and ternary REE treatments, respectively. Our result showed that the Ternary-Plus model and WHAM-F_{TOX} model are promising tools to account for the interaction of REEs in mixtures and could be used for their risk assessment.

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1. Introduction

Rare earth elements (REEs) include lanthanides, scandium and yttrium. Based on their atomic numbers they are further divided into two groups: light rare earth elements (LREEs) and heavy rare earth elements (HREEs). REEs are becoming a highly valuable commodity because of their increasing use in emerging technologies, especially renewable energy (Lambert and Ledrich, 2014). Many new mines are being opened in the US, Australia, and China due to a high demand for REEs. Moreover, lanthanides have been widely applied as fertilizers and animal feed stocks to promote growth (Wang and Liang, 2015; Skovran and Martinez-Gomez, 2015). As a result of these anthropogenic activities, a large quantity of REEs have been released into the ambient environment, posing potential ecological risks (Gonzalez et al., 2015). Although many studies have reported the toxicological effects of REEs on aquatic and terrestrial organisms, most of them focused on the evaluation and comparison of the toxic effect of individual REEs, ignoring mixture effects (Gonzalez et al., 2015, 2014; Herrmann et al., 2016). In reality, the combined pollution of REEs have been reported in sediment (Romero-Freire et al., 2018), water (Amyot et al., 2017), and mining-impacted soil (Chao et al., 2016), highlighting the relevance of studying REE mixture toxicity.

How to accurately quantify the pattern and intensity of the interactions between the different components is a daunting challenge for the assessment of mixture effects. Based on the meta-analysis of toxicity patterns of divalent metal mixtures, the occurrence of less-than-additivity (antagonism) or more-than-additivity (synergism) is of higher frequency than simple additivity (Feng et al., 2018; Norwood et al., 2003). For trivalent elements like REEs, having similar physiological properties and probably sharing common transport sites, antagonistic effects could be speculated when they exist simultaneously. Competitive effect of three lanthanides (La, Ce and Eu) on Sm were shown in algae (*Chlamydomonas reinhardtii*), all having similar estimated binding constants of $10^{6.8}$-$10^{7.0}$ M^{-1} (Tan et al., 2017). Nevertheless, there is still no clear interaction pattern of REEs in binary mixtures, not to mention ternary mixtures, which may include more complex interactions (Meyer et al., 2015). Consequently, there is a need for the development of multi-REE models that can qualitatively and quantitatively consider interactions and accurately predict mixture toxicity.

Traditionally, two reference models, Concentration Addition (CA) and Independent Action (IA), are applied for predicting the joint effect of mixtures containing metals with similar or dissimilar modes of action, respectively (Backhaus et al. (2000)). Mutual interactions of the toxic components in the mixture may affect their chemical activity or bioavailability, yet this was not considered at all while developing CA or IA model. As a consequence, they could not adequately predict mixture toxicity if one metal modifies the uptake of the other and subsequently affects the joint toxicity of these two metals (Vijver et al., 2011; Komjarova and Blust, 2009). A mathematical model, MIXTOX, was developed for detecting and quantifying deviations from the CA or IA model, estimating deviation parameters using a coherent data analysis procedure (Jonker et al., 2005). The MIXTOX model has shown suitable for determining the interactions of binary mixtures of metals (He et al., 2015; Robinson et al., 2017; Gong et al., 2019a), but few studies have focused on ternary mixtures (Traudt et al., 2017).

Compared to this mathematical model, a bioavailability-based model could help to better understand how interactions are induced in a mixture of metals and might also predict mixture toxicity better (Meyer et al., 2015). Several mechanistically-based bioavailability models have been proposed for predicting metal mixture toxicity, e.g., the Multi-metal Biotic Ligand Model (mBLM) and WHAM-F_{TOX}. The mBLM considers the competition among metals for biotic ligand sites by using binding constants derived from single metal toxicity data, assuming that the amount of metal binding to biotic ligands is determining toxicity (Jho et al., 2011; Versieren et al., 2014). As the binding constants of all individual metals are needed for applying the mBLM model, a large number of toxicity tests is needed for generating the required parameters. The WHAM-F_{TOX} model takes humic acid (HA) particulates as the proxy of binding sites and calculates the amount of metal binding to HA with a speciation model (WHAM VII), requiring fewer parameters than the mBLM model (Tipping and Lofts, 2013, 2015; Qiu et al., 2015). These bioavailability models have been shown to relatively accurately predict acute and chronic toxicity of metal mixtures to organisms (Iwasaki et al., 2015; Santore and Ryan, 2015; He and Van Gestel, 2015). However, the application of these mixture models was mainly for the prediction of binary mixture toxicity of divalent metal ions. Such models are still lacking for ternary mixture of trivalent metal ions.

For binary metal mixtures, antagonistic interactions between Ni and Co disappeared when exposure was related to body metal concentrations in *Enchytraeus crypticus* instead of concentrations in test solutions (He et al., 2015). When determining the joint effect of Hg and Se to *Caenorhabditis elegans* on the basis of concentrations in the exposure medium, additivity and antagonistic and synergistic interactions were observed. Antagonistic effects could, however, not be found on the basis of internal Hg/Se concentrations in the nematodes (Wyatt et al., 2016). The difference of identified interaction patterns based on different exposure levels may be explained by interactions at different steps in the intoxication process: bioavailability, uptake, toxicological and internal pathways (e.g., detoxification processes) (Vijver et al., 2011; Versieren et al., 2016). For the toxicity of a ternary mixture of Ni, Cu and Cd to *Lemna minor*, models were fitted based on internal and external concentrations simultaneously, showing that Ni-Cu-Cd competed for uptake, but once inside the plants only Cu-Cd shared a binding site (Gopalapillai and Hale, 2017). The use of internal concentrations already seems to integrate all interactions of the metals in solution and during their uptake into the organism. The determination and comparison of interactions on the basis of external and internal concentrations is helpful to determine where interactions happen and what is their magnitude.

In the present study, we aimed to examine the interaction of REEs in a ternary mixture, both qualitatively and quantitatively, and to search for a suitable model for the prediction of the joint effects of REE mixtures. Three mathematically-based models, the CA model, Ternary model, and Ternary-Plus model, were applied separately to interpret and predict toxicity and interactions of REE mixtures. Both free ion activity (external dose) and plant uptake (internal dose) of REEs were used to as exposure descriptors to help identifying at which steps during the intoxication process the interactions occur. It is hypothesized that interactions during uptake could be incorporated based on internal concentrations. The WHAM-F_{TOX} model was also applied to see if a bioavailability-based model could explain interactions and quantify the toxicity of REE mixtures.

2. Material and methods

2.1. Test solutions

The toxicity of Y, La, Ce, and their mixtures was examined in hydroponic cultures. The composition of Hoagland nutrient solution were slightly modified (Gong et al., 2019b) and used in the current study. Test solutions were prepared with three rare-earth chlorides ($YCl_3{\cdot}6H_2O$, $LaCl_3{\cdot}6H_2O$, and $CeCl_3{\cdot}7H_2O$) using the nutrient solution. A full factorial design was adopted for preparing the treatments (Y, La, Ce, Y-La, Y-Ce, La-Ce, and Y-La-Ce mixtures) (see Table S1 for mixture combinations). For all test solutions, the pH values were adjusted to 6.0 ± 0.2 one day before toxic exposure using 0.75 mg/L MES (active in the pH range of 5.5–6.7), diluted HCl, and diluted NaOH when necessary.

2.2. Plant bioassays

The 4 days plant root elongation tests were conducted following a standard protocol with slight modification (Gong et al., 2019b). The wheat *Triticum aestivum* L., representative of crops, was tested. For the exposure experiments, four uniform wheat seedlings (~1.5 cm) were transferred into a glass beaker filled with 250 mL of test solution. Each treatment was repeated three times. During the exposure, the test solution was changed daily to reduce the potential influences of plant growth on the concentrations of REEs and solution pH. After 4 days exposure, the longest root of each wheat seedling was recorded. Relative root elongation (RRE, %) was used as an indicator of toxicity:

$$\mathrm{RRE}(\%) = \frac{L_{\mathrm{REEs}} - L_{\mathrm{initial}}}{L_{\mathrm{control}} - L_{\mathrm{initial}}} \times 100 \quad (1)$$

where $L_{initial}$ (cm) is the initial root length, L_{REEs} (cm) is the root length of the seedlings in a specific treatment, and $L_{control}$ (cm) is the root length in the control treatment. The collected wheat roots were washed with 0.02 M Na_2EDTA solution to remove surface-bound metals. And then, the roots were flushed with deionized water, dried at 80 °C, and digested with *aqua regia* for further metal analysis.

2.3. Chemical measurements

Solution pH before and after the bioassay was measured using a pH meter (pH 1120x, Mettler Toledo, Switzerland). The average values were reported and used for speciation calculation. Dissolved concentrations of REEs and other coexisting cations in the test solutions and the concentrations of REEs in the digestive solution of plant roots were analyzed by ICP-OES (iCAP7600, Thermo Fisher, USA; detection limit: 0.05 mg L^{-1}) and if necessary ICP-MS (iCAPQ, Thermo Fisher, USA; detection limit: 0.0005 μg L^{-1}). To maintain the quality of analysis, a calibration standard (AccuStandard, Agilent Solutions, USA) and a reagent blank were run every 30 samples. Free Y^{3+}, La^{3+}, and Ce^{3+} activities in the test solutions were calculated using the Windermere Humic Aqueous model (WHAM VII) software package (Tipping et al., 2011). The solution pH, temperature (20 °C), partial pressure of CO_2 ($10^{-3.5}$ atm), and the measured concentrations of dissolved elements were the required input parameters for speciation calculation. The activities of free Y^{3+}, La^{3+} and Ce^{3+} and measured concentrations of REEs in plant roots were used as exposure dose for data analysis in the present study.

2.4. Model formulation

2.4.1. Log-logistic regression

The log-logistic model was used to describe the dose-response relationships of exposure to a single rare earth element *i*.

$$\mathrm{RRE}(\%) = \frac{100}{1 + (\frac{c_i}{\mathrm{EC50}_i})^{\beta_i}} \quad (2)$$

where RRE is relative root elongation (%), c_i is the exposure concentration of REE *i* (free ion activity in test solution (μM); concentration in plant roots (mg kg^{-1})), $EC50_i$ is the effective concentration of REE *i* triggering 50 % effect, βi is the slope parameter of REE *i*.

2.4.2. Mathematical models for describing mixture effects

2.4.2.1. Concentration addition (CA) model. CA is traditionally applied for predicting mixture toxicity of chemicals that are assumed to have similar modes of action (MoA) and do not interact with each other (Jonker et al., 2005). The properties of lanthanides are very similar, justifying the use of CA as the reference model. The concept of CA model can be mathematically expressed as:

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC50}_i \times (\frac{100 - \mathrm{RRE_{mix}}}{\mathrm{RRE_{mix}}})^{1/\beta_i}} = 1 \quad (3)$$

where $EC50_i$ and β_i are the dose-response parameters for each REE when applied individually, c_i is the dose of each mixture component, RRE_{mix} is the predicted mixture effect. The CA model was applied for predicting the joint effect of binary mixtures (e.g. Y-La) and ternary mixtures (Y-La-Ce) of REEs. To find the CA-predicted RRE_{mix}, Eq. (3) were numerically solved using the generalized-reduced-gradient-iterative solver function (JMP 16.0, SAS Institute).

2.4.2.2. Quantifying deviation from CA model. Eq. (3) was rewritten in terms of joint effect of REEs in the mixtures with incorporation of the function G to describe the degree of deviation from the CA model.

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC50}_i \times (\frac{100 - \mathrm{RRE_{mix}}}{\mathrm{RRE_{mix}}})^{1/\beta_i}} = \exp(\mathrm{G}) \quad (4)$$

The deviation function G is described by the parameter a ($a > 0$ indicates antagonism, $a = 0$ additivity, and $a < 0$ synergism) and relative contribution of the toxicity of each REE z_i (calculated by toxic unit of each REE, TUx_i).

$$\mathrm{G}(z_1, \ldots, z_n) = a\prod_{i=1}^{n} z_i \quad (5)$$

$$z_i = \frac{\mathrm{TUx}_i}{\sum_{i=1}^{n} \mathrm{TUx}_i}$$

where

$$\mathrm{TUx}_i = \frac{c_i}{\mathrm{ECx}_i} \quad (6)$$

2.4.2.3. Binary model incorporating deviation. For predicting the joint effect of binary mixtures of REEs (e.g. for Y-La), Eq. (5) was rewritten as Eq. (7), a_{Y-La} indicates the interaction between Y and La.

$$\mathrm{G}(z_Y, z_{La}) = a_{Y-La} \bullet z_Y \bullet z_{La} \quad (7)$$

To find the Binary model-predicted RRE_{mix} and the parameter a_{Y-La}, the binary deviation function Eq. (7) was substituted into Eq. (4) and fitted by minimizing the sum of squared residuals of predicted and observed toxicity data of binary mixtures. Simultaneously, the same data analyzing process was applied for the binary mixtures of Y-Ce and La-Ce, and the estimated parameters a_{Y-La}, a_{Y-Ce}, and a_{La-Ce} were held constant in the following data analysis using the Ternary model and Ternary-Plus model.

2.4.2.4. Ternary model incorporating deviation of binary mixtures. The joint effect of the ternary mixture (Y-La-Ce) was predicted by incorporating the defined interactions of the binary mixtures of Y-La, Y-Ce and La-Ce. To find the Ternary model-predicted RRE_{mix}, the ternary deviation function Eq. (8) was substituted into Eq. (4) and fitted by minimizing the sum of squared residuals of predicted and observed toxicity data of ternary mixtures.

$$\mathrm{G}(z_Y, z_{La}, z_{Ce}) = a_{Y-La} \bullet z_Y \bullet z_{La} + a_{Y-Ce} \bullet z_Y \bullet z_{Ce} + a_{La-Ce} \bullet z_{La} \bullet z_{Ce} \quad (8)$$

2.4.2.5. Ternary-Plus model incorporating both binary and ternary deviations. The Ternary model was further developed considering the interaction among Y, La, and Ce, with parameter $a_{Y-La-Ce}$ indicates the deviation of from measured toxicity for the ternary mixture. To find the Ternary-Plus model-predicted RRE_{mix} and the parameter $a_{Y-La-Ce}$, the ternary deviation function Eq. (9) was substituted into Eq. (4) and fitted by minimizing the sum of squared residuals of predicted and observed toxicity data of ternary mixtures.

$$G(z_Y, z_{La}, z_{Ce}) = a_{Y-La} \cdot z_Y \cdot z_{La} + a_{Y-Ce} \cdot z_Y \cdot z_{Ce} + a_{La-Ce} \cdot z_{La} \cdot z_{Ce} + a_{Y-La-Ce} \cdot z_Y \cdot z_{La} \cdot z_{Ce} \quad (9)$$

For determining and quantifying where interactions occur and their intensity, all models were fitted relating toxicity to free ion activity in test solutions (μM) and to concentrations taken up in plant roots (mg kg^{-1}).

2.4.3. Bioavailability models for describing mixture effects

The WHAM-F_{TOX} model describes the combined toxic effects of metal i on organisms through the toxicity function (F_{TOX}), which is a linear combination of the products of organism-bound metal (ν_i) and a toxic potency coefficient (α_i) for each metal and H^+ (Tipping et al., 2019).

$$F_{TOX} = \sum_{i=1}^{n} \alpha_i \times \nu_i = \alpha_Y \times \nu_Y + \alpha_{La} \times \nu_{La} + \alpha_{Ce} \times \nu_{Ce} + \alpha_H \times \nu_H \quad (10)$$

Eq. (10) was used to link the response of test organisms (e.g. RRE%) to F_{TOX}, expressed as:

$$RRE(\%) = \frac{100}{1 + \left(\frac{F_{TOX}}{F_{TOX50}}\right)^{\beta}} = \frac{100}{1 + \left(\frac{\alpha_Y \times \nu_Y + \alpha_{La} \times \nu_{La} + \alpha_{Ce} \times \nu_{Ce} + \alpha_H \times \nu_H}{F_{TOX50}}\right)^{\beta}} \quad (11)$$

F_{TOX50} is the F_{TOX} causing 50 % toxic effect. The value of ν_i for each metal is calculated with the Windermere Humic Aqueous Model, using a default concentration of HA of 5.0×10^{-6} g L^{-1} that is sufficiently low to have no impact on REE speciation in the test solutions. The values of α_i, β, and F_{TOX50} were estimated by fitting the model to all toxicity data.

In the present study, for all the applied models, the Model deviation ratio (MDR, model predicted value/observed value) and Root Mean Squared Error (RMSE) were calculated as indicators of model deviation.

3. Results and discussion

3.1. Individual toxicity of REEs

For the toxicity of the individual Y, La and Ce to wheat root elongation, EC50 s values were 0.61, 0.81 and 0.65 μM on the basis of free ion activity in test solutions, and 431, 940 and 607 mg kg^{-1} on the basis of root uptake concentrations, respectively (Fig. S1). Based on the calculated EC50 s, it can be seen that Y was the most toxic element, followed by Ce and La (Table S2).

As Y belongs to the HREE, while La and Ce are LREE, this suggests that HREE are more toxic to *Triticum aestivum* L. than LREE. The heavier REE Tb was found more cytotoxic than the lighter La, with the difference in toxicity resulting from the different binding affinity to the K^+ channel (Wang et al., 2017). However, opposite results were also reported: the toxicity of lanthanides to the amphipod *Hyalella azteca* exhibited a decreasing trend with increasing atomic number from La to Er, but Tm and Lu were most toxic (Borgmann et al., 2010). There is still no coincident accumulation and toxicity trend of REEs with different atomic numbers, it may be both test conditions and test species dependent.

3.2. Toxicity of REEs in binary mixtures

Based on the estimated EC50 s for the single REEs, joint effects of the binary REE mixtures on relative root elongation were predicted with the CA model. The predicted effects of Y-La, Y-Ce and La-Ce treatments closely correlated with the observed values, with R^2 and MDR (model deviation ratio) values of 0.95 and 0.92, 0.93 and 0.91, and 0.93 and 0.87 when based on free ion activity. These values were 0.91 and 0.85, 0.69 and 1.09, and 0.76 and 0.87, respectively, when expressed as root uptake concentration (Table 1, Table S3, and Fig. S2).

As reflected from the obtained R^2 and MDR, the CA model explained most of the variations in binary mixture toxicity, proving that the assumption of CA is applicable for REEs, that is, the chemicals in the mixture have a similar mode of action (Cedergreen et al., 2008). It has been suggested that lanthanides could be considered as a uniform group of elements with similar mode of action (Blinova et al., 2018). Compared to the IA model, the CA model therefore may serve as a more suitable reference model for REE mixtures.

The Binary model was applied to predict the joint effects incorporating the interactions of the REEs in the binary mixtures. The deviation function for binary mixtures was quantified and shown in Table 1. Estimated deviation parameters a_{Y-La}, a_{Y-Ce}, a_{La-Ce} were 0.008, 0.759 and −0.640 on the basis of free ion activity, and −0.0477, −0.237 and −0.252 on the basis of root uptake concentrations. Compared to the CA model, there was no significant improvement of model fitting, with obtained p value > 0.05 and MDR value not obviously differed from that of CA model (Fig. S2 and Table S3). This indicates there is no obvious interaction between the REEs in the binary mixtures, so mainly showing an additivity effect. For the mixture toxic effect of lanthanides on marine microalgae (*Skeletonema costatum*), also an additive mode of action was found (Tai et al., 2010). However, inconsistent findings were reported for the interaction pattern of REE mixtures (Tan et al., 2017; Romero-Freire et al., 2019). The difference among antagonistic, synergistic or additive impacts may depend on chemical species, exposure concentrations, organisms and toxicological endpoint examined (Vijver et al., 2011, 2010).

3.3. Toxicity of REEs in ternary mixtures

Join effects of the REE ternary mixture were fitted with the CA, Ternary and Ternary-Plus models, with free ion activity (external) and root uptake concentration (internal) of REEs as expressions of exposure. Figs. 1 and 2 show the relationship between the predicted and observed mixture toxicity, with estimated R^2 and p values. In general, the goodness of fit did not differ when considering the identified binary interactions, while it improved significantly with incorporation of the ternary interactions of Y-La-Ce, with MDR values of 0.75, 0.77, and 0.93 based on free ion activity and 0.80, 0.81, and 0.91 based on root uptake concentration for CA, Ternary, and Ternary-Plus model, respectively (Table S3).

When fitting toxicity data of all treatments together, the estimated parameter $a_{Y\text{-}La\text{-}Ce}$ was positive, with values of 25.4 and 14.6 on the basis of free ion activity and plant uptake concentrations, respectively (Tables 2 and 3). The result indicates antagonistic interactions among the REEs in the ternary mixture, although additivity was found for binary mixtures. In previous studies, the interaction patterns of ternary metal mixtures were not always in accordance with that of binary mixtures. For the mixture toxicity of Cu, Cd and Pb to cucumber *Cucumis sativus*, the ternary combination showed antagonism even though additive or synergistic interactions were found for their binary combinations (An et al., 2004). The alleviation or elevation of the joint effects induced by the coexistence with other metals can partly be attributed to the competitive or anti-competitive interactions during the accumulation and intoxication process (Vijver et al., 2011; Norwood et al., 2013; Van Ginneken et al., 2015). The competitive effect means that the presence of one metal can inhibit the binding of the other metal on uptake or toxic action sites, resulting in reduced joint toxic effect. While, anti-competitive effect indicates the opposite case where the binding of one metal to uptake or toxic action sites is enhanced by the other metal, resulting in increased joint toxic effect (Wyatt et al., 2016). The bioaccumulation of individual metals in shoots of *Cucumis sativus* was inhibited in the ternary mixtures by the presence of other metals (An et al., 2004). Similarly, Zn and Co inhibited each other's uptake in *Triticum aestivum* (Wang et al., 2013). The bioaccumulation of elements was influenced in exposures to mixtures compared with individual metal exposures (Norwood et al., 2013). These findings prove that the

Table 1
Results of fitting data for the toxicity to *Triticum aestivum* root elongation of binary mixtures of the rare earth elements Y, La and Ce with the Concentration Addition model (CA) and the Binary model based on free ion activity and root uptake concentrations, respectively. *a* is the estimated parameter indicating the deviation of binary mixture effect from CA model, *p* indicates the outcome of the likelihood ratio test.

Exposure expressed as	Models	Y-La		Y-Ce		La-Ce		Interaction pattern
Free ion activity	CA	R^2	0.95	R^2	0.93	R^2	0.93	Additivity
	Binary	R^2	0.95	R^2	0.93	R^2	0.94	
		$a_{Y\text{-}La}$	0.008	$a_{Y\text{-}Ce}$	0.759	$a_{La\text{-}Ce}$	−0.640	
		p	0.98	p	0.053	p	0.076	
Root uptake concentration	CA	R^2	0.91	R^2	0.69	R^2	0.75	Additivity
	Binary	R^2	0.91	R^2	0.70	R^2	0.77	
		$a_{Y\text{-}La}$	−0.0477	$a_{Y\text{-}Ce}$	−0.237	$a_{La\text{-}Ce}$	−0.252	
		p	0.62	p	0.72	p	0.52	

interactive effect happened during the accumulation process.

The estimated value of $a_{Y\text{-}La\text{-}Ce}$ was almost two-fold lower when based on internal concentrations than external exposure levels. Consistently, the standardized regression coefficients, which indicate the binary interaction was approximately 3-fold smaller for Ni-Cu and Ni-Cd on the basis of internal concentrations than that for external dose, the effect of Cd, Cu and Ni were additive at biotic ligands (Gopalapillai and Hale, 2017). Mixture interactions Cd, Cu and Pb in isopods *Asellus aquaticus* disappeared when growth and mortality were related to body concentrations instead of external exposure concentrations (Van Ginneken et al., 2015). The internal concentration apparently already takes into account interactions occurring during the accumulation process, so the quantified deviation based on internal concentrations could indicate the interaction during the intoxication process. In the present study, even when using internal concentration as exposure level, the deviation from CA was significant ($a_{Y\text{-}La\text{-}Ce} > 0$, $p < 0.01$), demonstrating that the interaction of the REEs took place during both the accumulation and intoxication process. Overall, internal concentrations serve as a better indicator of mixture toxicity and are helpful to reveal at which level interactions in metal mixtures occur.

In the present study, Ce, La and Y were set as independent variables to evaluate their contribution to the ternary interaction. The estimated values of the deviation function ($a_{Y\text{-}La\text{-}Ce}$) from CA with varying concentrations of Y, La and Ce are shown in Tables 2 and 3, expressed on the basis of free ion activity and root uptake concentrations, respectively. Overall, the interaction pattern of the REE ternary mixture did not change with the variation of the independent variables, always quantified as antagonism. The interaction intensity showed a decrease with the increasing value of the independent variables, and then increased at the highest level. Compared to external concentrations, the effect of the independent variables on the interaction was weaker when related to internal concentrations. It has been showed that the degree of joint effect of binary mixtures could be influenced by the ratio of individual chemicals in a mixture, e.g. Cu-Zn for tilapia and Cu-Ni for barely (Wang et al., 2018; Obinna Obiakor and Damian Ezeonyejiaku, 2015). For ternary mixtures, similar trends were also observed when assessing the effect of Ni on the interactions between Cd and Cu; from the response surfaces it can be seen that the elevated Ni level caused a reduced antagonistic interaction when related to tissue metal concentrations (Gopalapillai and Hale, 2017). For the toxicity of ternary mixtures of Cd, Cu and Ni to *Daphnia magna*, joint effects of Cd-Ni and Cd-Cu ranged from less-than-additive to approximately additive to more-than-additive toxicity with increasing concentrations of single Cu and Ni (Traudt et al., 2017). It was supposed that biologically significant deviations from a reference model are most likely to occur when toxicity from a few components dominates a multi component mixture (McCarty and Borgert (2006)). A straight-forward prediction of which mixtures will give rise to deviations from the reference model and at what magnitude is not possible. In the present study, we quantified the deviation parameter with the variation of Ce, La and Y concentrations separately to identify the relative effect of each element. With increase of Ce, La and Y concentrations, $a_{Y-La-Ce}$ ranged from 10.2 to 63.7, from 22.2 to 48.4, from 21.0 to 33.3 when related to free ion activity (Table 2), and from 6.05 to 49.4, from 9.55 to 39.3, from 8.57 to 19.6 when expressed as root uptake concentrations (Table 3), respectively. Ce had the strongest influence, followed by La and Y. This rank order of the magnitude of influence on mixture interactions is consistent with the binding affinity of the individual elements, with *Enchytraeus crypticus* accumulating almost equal amounts of La and Ce, but much lower amounts of Y at the same exposure concentration. In the BLM concept, the binding affinity is used to quantitatively express the competition of metal ions and cations for biotic ligands, meaning that cations/ions with high binding affinity have a stronger mitigating effect (Di Toro et al., 2001; Thakali et al., 2006).

To sum up, mixture effects could be overestimated when using the CA model based on toxicity data of individual REEs. The importance of considering ternary interaction in the risk assessment of REE mixtures should be emphasized in future studies.

3.4. Applicability of WHAM-FTOX model for predicting mixture toxicity

The WHAM-F_{TOX} model was fitted to all toxicity data of the single, binary and ternary exposures to the three REEs. The toxicity coefficients (α_i) obtained were 2.77, 3.28 and 3.19 for Y, La and Ce separately, indicating all three REEs contributed significantly to the toxicity in the mixtures. The observed RRE% was plotted against the calculated F_{TOX} (Fig. 3), giving R^2 values of 0.94, 0.88, 0.85, and 0.77 and MDR values of 1.08, 1.11, 0.94, and 1.06 for individual REE, binary mixture, ternary mixture, and all toxicity data together, respectively. This shows that both binary and ternary mixture toxicity of REEs can be well predicted with the WHAM-F_{TOX} model.

Previous studies have tested the applicability of the WHAM-F_{TOX} model for the prediction of the mixture toxicity of metals, but mainly for binary mixtures of divalent metal ions (Tipping and Lofts, 2013; Qiu et al., 2015; He and Van Gestel, 2015). It has been shown the net root elongation of *Lemna minor* was highly correlated with amount of Cu bound to particulate humic acid (Antunes et al., 2012). Compared to total metal concentrations, free metal ion activities, as a cumulative criterion unit, the amount of metal bound to humic acid (ν_i) are better predictors of metal mixture toxicity to macroinvertebrates (Iwasaki et al., 2013). Reasonable fits of the WHAM-F_{TOX} model were obtained for different metal mixture exposures and different species, including daphnids, lettuce and trout (Tipping and Lofts, 2015). Toxicity data for a ternary mixture of Cu, Zn and Cd to zebra mussel showed a very good fit with the WHAM-F_{TOX} model (Tipping and Lofts, 2013). Consistent with our results, these findings support the assumption of the WHAM-F_{TOX} model that humic acid could act as a surrogate of non-specific binding sites for describing metal mixture toxicity. A noteworthy feature is that it chemically incorporates the competition of metals with other cations and competition among metals at the binding sites by using metal ions binding by humic acid as a proxy (Stockdale et al., 2010). Hence, competing interactions in ternary-metal mixtures could

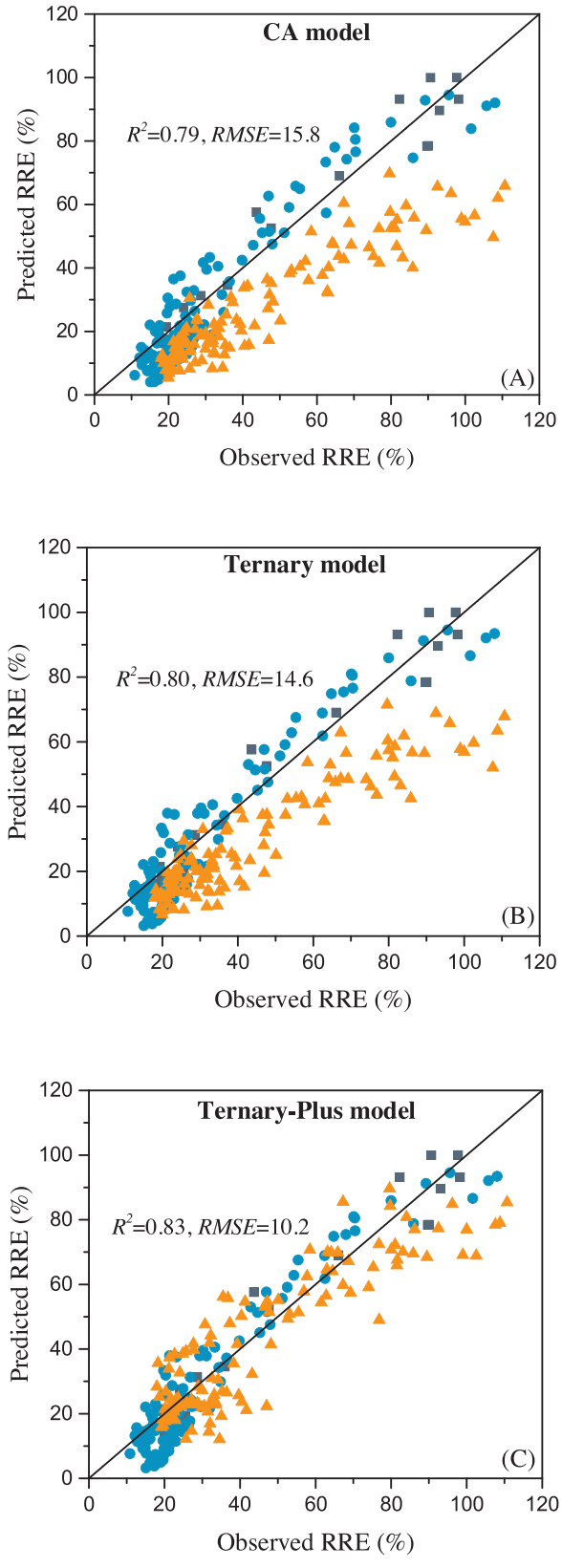

Fig. 1. Relationship between the predicted and observed relative root elongation (RRE %) of *Triticum aestivum* exposed to single (Y, La, Ce) (square points), binary mixtures (Y-La, Y-Ce, La-Ce) (cycle points) and ternary mixture (Y-La-Ce) (triangle points) based on external concentrations (free ion activity). Data were fitted with (A) Concentration Addition model (CA), (B) Ternary model, incorporating the deviations for the binary mixtures ($a_{Y\text{-}La}$, $a_{Y\text{-}Ce}$, $a_{La\text{-}Ce}$), (C) Ternary-Plus mode, incorporating both the deviations for the binary mixtures and the deviation for the ternary mixture ($a_{Y\text{-}La\text{-}Ce}$). The solid lines represent 1:1

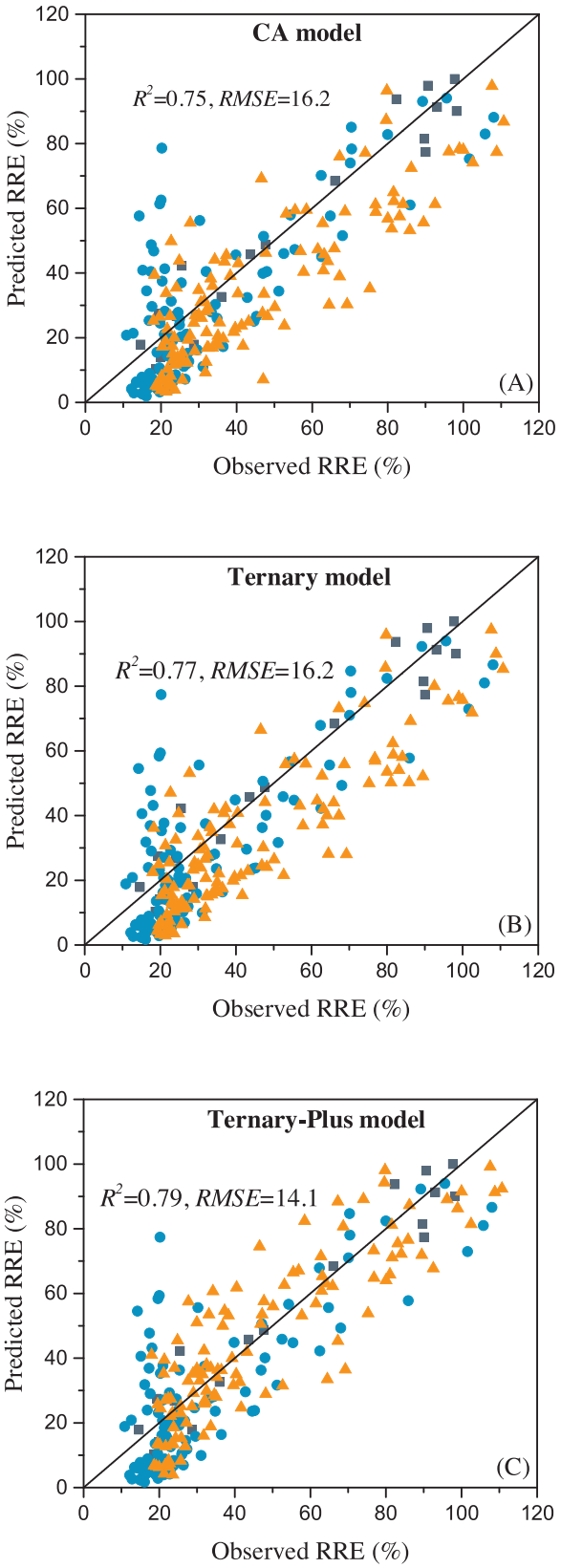

Fig. 2. Relationship between the predicted and observed relative root elongation (RRE %) of *Triticum aestivum* L. exposed to single (Y, La, Ce) (square points), binary mixtures (Y-La, Y-Ce, La-Ce) (cycle points) and ternary mixture (Y-La-Ce) (triangle points) based on internal concentrations (root uptake). Data was fitted with (A) Concentration Addition model (CA), (B) Ternary model, incorporating the deviations for the binary mixtures ($a_{Y\text{-}La}$, $a_{Y\text{-}Ce}$, $a_{La\text{-}Ce}$), (C) Ternary-Plus mode, incorporating both the deviations for the binary mixtures and the deviation for the ternary mixture ($a_{Y\text{-}La\text{-}Ce}$). The solid lines represent 1:1

Table 2
Results of fitting data for the toxicity to *Triticum aestivum* root elongation of ternary mixtures of the rare earth elements Y, La and Ce with Concentration Addition model (CA), Ternary model (with fixed deviation parameters $a_{Y\text{-}La}$, $a_{Y\text{-}Ce}$, $a_{La\text{-}Ce}$ estimated from binary mixture treatment), Ternary-Plus model (incorporating ternary deviation parameter $a_{Y\text{-}La\text{-}Ce}$) based on free ion activity of REEs.

Y + La + (Ce)	Ce (μM)						
			0.25	0.5	1.0	2.0	4.0
	CA	R^2	0.72	0.71	0.82	0.93	0.93
	Ternary	R^2	0.74	0.74	0.84	0.93	0.93
		p	0.03	0.01	< 0.01	< 0.01	< 0.01
	Ternary-Plus	R^2	0.89	0.90	0.92	0.94	0.94
		$a_{Y\text{-}La\text{-}Ce}$	63.7	37.7	22.9	10.2	27.9
		p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Y + Ce + (La)		La (μM)					
			0.25	0.5	1.0	2.0	4.0
	CA	R^2	0.77	0.84	0.73	0.84	0.88
	Ternary	R^2	0.79	0.84	0.73	0.84	0.89
		p	0.02	0.05	0.42	0.78	0.69
	Ternary-Plus	R^2	0.86	0.91	0.88	0.90	0.89
		$a_{Y\text{-}La\text{-}Ce}$	48.4	26.6	30.0	22.2	30.4
		p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
La + Ce + (Y)	Y (μM)						
			0.25	0.5	1.0	2.0	4.0
	CA	R^2	0.80	0.75	0.72	0.87	0.92
	Ternary	R^2	0.80	0.75	0.74	0.88	0.92
		p	0.13	0.62	0.08	0.02	0.03
	Ternary-Plus	R^2	0.87	0.86	0.86	0.90	0.92
		$a_{Y\text{-}La\text{-}Ce}$	33.3	28.8	29.9	21.0	32.0
		p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Overall	CA	R^2	0.79				
	Ternary	R^2	0.80				
		p	< 0.01				
	Ternary-Plus	R^2	0.83				
		$a_{Y\text{-}La\text{-}Ce}$	25.4				
		p	< 0.01				

Table 3
Results of fitting data for the toxicity to *Triticum aestivum* root elongation of ternary mixtures of the rare earth elements Y, La and Ce with Concentration Addition model (CA), Ternary model (with fixed deviation parameters $a_{Y\text{-}La}$, $a_{Y\text{-}Ce}$, $a_{La\text{-}Ce}$ estimated from binary mixture treatment), Ternary-Plus model (incorporating ternary deviation parameter $a_{Y\text{-}La\text{-}Ce}$) based on root uptake of REEs.

		Ce (μM)					
			0.25	0.5	1.0	2.0	4.0
	CA	R^2	0.82	0.79	0.80	0.72	0.89
	Ternary	R^2	0.83	0.82	0.83	0.73	0.89
		p	0.03	< 0.01	< 0.01	0.51	0.12
	Ternary-Plus	R^2	0.85	0.86	0.87	0.73	0.89
		$a_{Y\text{-}La\text{-}Ce}$	11.6	13.7	15.7	6.05	49.4
		p	< 0.01	< 0.01	< 0.01	0.19	< 0.01
Y + Ce + (La)		La (μM)					
			0.25	0.5	1.0	2.0	4.0
	CA	R^2	0.72	0.79	0.84	0.79	0.88
	Ternary	***R2***	0.75	0.80	0.86	0.80	0.88
		p	0.03	0.03	< 0.01	0.03	0.03
	Ternary-Plus	R^2	0.81	0.85	0.91	0.83	0.86
		$a_{Y\text{-}La\text{-}Ce}$	39.3	17.6	16.2	10.9	9.55
		p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
La + Ce + (Y)		Y (μM)					
			0.25	0.5	1.0	2.0	4.0
	CA	R^2	0.77	0.83	0.72	0.84	0.91
	Ternary	R^2	0.79	0.83	0.74	0.84	0.91
		p	0.01	0.02	0.02	0.14	0.06
	Ternary-Plus	R^2	0.82	0.84	0.79	0.84	0.91
		$a_{Y\text{-}La\text{-}Ce}$	19.6	11.1	13.5	8.57	19.5
		p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Overall	CA	R^2	0.75				
	Ternary	R^2	0.77				
		p	0.22				
	Ternary-Plus	R^2	0.79				
		$a_{Y\text{-}La\text{-}Ce}$	14.6				
		p	< 0.01				

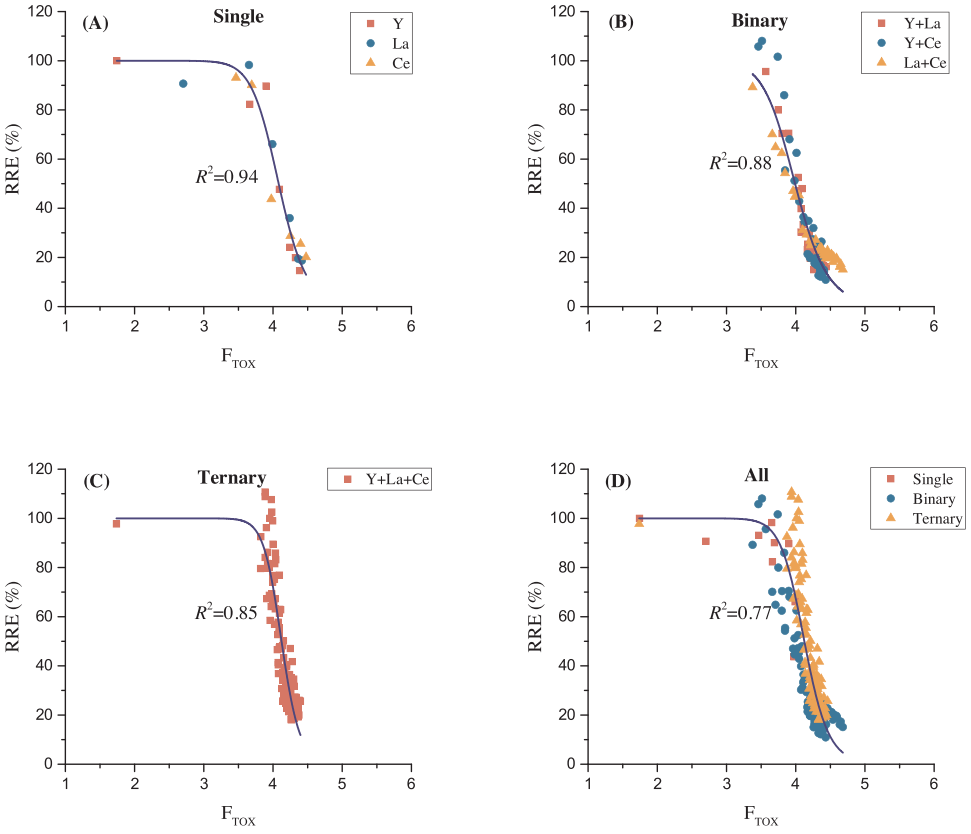

Fig. 3. Correlation of the relative root elongations (RRE%) of *Triticum aestivum* L. under the exposure to individual REE (Y, La, Ce) (A), binary mixtures of REE (Y-La, Y-Ce, La-Ce) (B), ternary mixtures of REE (Y-La-Ce) (C) and all treatments together (D) to F_{TOX}. The symbols indicate the mean value of observed toxicity data (n = 3), the solid lines represent the fitting of the WHAM-F_{TOX} model for toxicity data of single and mixture exposure, with estimated R^2 value showing the goodness of fit.

be incorporated in bioavailability-based toxicity models. In general, the predictive ability of the bioavailability-based model for the mixture toxicity of REEs to wheat is almost equal to that of the mathematically-based model. But for the prediction of ternary mixture toxicity, the data fitting process of WHAM-F_{TOX} is simpler than with the Ternary-Plus model, requiring fewer parameters to be estimated.

4. Conclusions

Identifying and quantifying the potential interactions of REEs in mixtures is crucial for an accurate risk assessment. The present study focused on the evaluation of joint effects of Y, La and Ce on the root elongation of wheat. Strong antagonism was observed for the ternary mixture and significant improvement of the predictive ability was observed when ternary mixture interactions were considered. Mitigated deviation from the CA model was observed when toxicity was related to root uptake concentrations (internal dose) instead of free ion activity (external dose), indicating possible competing effects of REEs during uptake and intoxication processes. The developed bioavailability-based models mainly rely on the assumption of metal-metal competition for binding sites on the organisms. Hence, by definition these models are applicable for predicting mixture toxicity of metals with antagonistic interactions but not for synergistic interactions. A future challenge is to reveal the mechanism behind these antagonistic and synergistic interactions. Although the mathematical model (Ternary-plus model) and the bioavailability-based model (WHAM-F_{TOX}) were equally valid in predicting REE mixture toxicity in the present study, but the latter model may show its superiority in predicting mixture effects under different exposure media. In other words, the mathematical model and derived parameters can just be used to quantify mixture toxicity in a specific exposure condition, while the mechanistic-based WHAM-F_{TOX} has the potential to reconcile variations in REE mixture toxicity across different exposure scenarios. Future research efforts should be made to develop models that can be applied for the toxicity prediction of REE mixtures in real contaminated soils with varying properties.

Authors contribution statement

HQ and EH conceived the idea and designed the experiment; BG performed the experiment; EH and HQ wrote the manuscript; CVG, JR, YT, XH, XX, ML, and RQ revised the manuscript; HQ, EH, and RQ raised the funding.

Declaration of Competing Interest

There is no competing interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.121940.

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